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**13. ABSTRACT** *(Maximum 200 Words)*

Angiogenesis, the formation of new blood vessels from existing vessels, is a process critical to many normal physiologic processes. However, uncontrolled capillary growth can have dramatic consequences, as seen in the rapid growth of well-vascularized tumors such as breast carcinoma. Indeed, failure to induce a new blood supply limits the size of a tumor dramatically. It is clear that controlling angiogenesis could have a major impact on treatment of breast cancer, the most common cancer in American women.

Using a PCR-based screen (Representational Difference Analysis) we have identified several genes that are involved in formation of new blood vessels and that are upregulated in cultured capillary endothelial cells (EC) forming tubes (neo-capillaries) in collagen gels, but not in EC growing in monolayers. ESM-1 is a secreted molecule that may be related to chondroitin sulfates. It is highly expressed in several tumors and appears to be regulated by the vascular endothelial growth factor, VEGF. Beta-ig-H3 is an extracellular matrix molecule involved in cross-linking of collagen fibers. Reducing its expression using antisense molecules blocks in vitro capillary formation. NrCAM is a "neural" adhesion molecule and is highly upregulated on tube-forming EC. We speculate that it may have a role in guidance of new vessels. HESR1 is a bHLH transcription factor that appears to control the switch from quiescence to migration and proliferation. It is downstream of the notch receptor and can suppress transcription of the VEGF receptor gene VEGFR2. Blocking HESR1 induction with antisense blocks tube formation.

By identifying genes differentially expressed during angiogenesis, and designing effective molecular therapies that reduce their expression and thereby reduce capillary growth, we hope to identify future targets for specific therapy aimed at blocking growth of solid tumors.

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## Table of Contents

## Introduction

If a growing tumor fails to establish a blood supply then its growth will be limited to a diameter of 1-2 mm. Similarly, if the vasculature of a tumor is destroyed, that tumor will die by necrosis. The degree of new blood vessel growth is a strong prognostic indicator in breast carcinoma as well as many other tumors. In this project we are identifying genes critical to new capillary formation and investigating molecular mechanisms for regulating their expression. The principal cellular component of blood vessels is the endothelial cell (EC). We have established culture systems for analyzing EC growth and differentiation and have used the technique of Representational Difference Analysis to identify genes expressed in tube-forming EC but not in cells growing in monolayers. We have characterized several of these genes and determined their expression and role in angiogenesis.

Our expectation is that characterization of these important regulatory proteins may prove useful as therapeutic targets for the prevention of new blood vessel growth into breast tumors.

## Body

In our previous reports we detailed our analysis of genes identified at 18 hours of culture as being present in tubes but not monolayers. In these screens we used both a high stringency for generating the DP3, namely 1:400,000, as well as a lower stringency (1:800) for generating DP2. The full protocol and the results we obtained are now in press and the MS is attached as an appendix.

It is our belief that the collagen gel model has probably yielded all the useful data it can. The problem with the assay is that it only models some aspects of new vessel formation. In this assay cells are randomly seeded in the gel and then "coalesce" into a network, at which time rudimentary lumen formation occurs. This is somewhat reminiscent of vasculogenesis but fails to model the sprouting phase of angiogenesis. For this reason we have optimized a new procedure where EC are grown on Cytodex beads, which are then embedded in fibrin gels. In the presence of growth factors the EC reproducibly generate stable, long-term, anastomosing networks of capillaries with patent lumens.

We have optimized gel pH (which affects rigidity), growth factor concentrations (which affects vessel length and diameter), bead concentration (which affects degree of anastomosis and complexity of the network) and gel composition (the presence of fibronectin helps sprouting). We use fibroblasts to condition the medium, presumably with growth factors such as angiopoietin-1 that stabilize newly-formed vessels. Interestingly, the capillaries are not invested with support cells, but do appear to be stabilized by fibroblast-derived factors, suggesting a direct effect rather than an indirect effect through pericytes as has been proposed in vivo. Directly embedding EC in fibrin gels does not yield such a complex, anastomosing network, which probably reflects the need of EC to go through the full program of sprouting, migration, alignment and tube formation, rather than being forced to "coalesce" into a vessel from an initial random distribution of cells in the gel.

*Task 1. Establish optimal conditions for monolayer and 3D collagen gel cultures*

- completed and detailed in previous reports

*Task 2. Generate high quality cDNA pools for RDA*

- completed and detailed in previous reports

*Task 3. Generate high quality cDNA libraries for screening*

- completed and detailed in previous reports

*Task 4. Use RDA to generate difference products*

- completed and detailed in previous reports

*Task 5. Block difference product expression using antisense oligonucleotides*

- *analyze sequence of target gene - select 20-25 oligonucleotides*
  Several have been tested (see MS in appendix)
- *perform dose-response analysis in tube-formation assay*
  Completed for βIg-H3, ESM-1 and NrCAM (see MS in appendix)
- *perform dose-response analysis in new angiogenesis assays*
  Ongoing for HESR1, βIg-H3, ESM-1 and NrCAM
- *repeat for each difference product of interest*
  Ongoing

*Task 6. Block difference product expression using dominant negative proteins*

- We are currently testing dominant negative versions of several notch and notch ligand proteins as we have recently shown that HESR1 is a downstream target of notch signaling in EC. Both notch 3 extracellular domain and jagged-1 extracellular domain have been shown to block tube formation in our assays.

## Key Research Accomplishments in years 1-3

- Establishment of culture conditions for EC tube formation
- Generation of cDNA libraries for monolayer and tube cultures
- Completion of first round of RDA
- Identification of four known genes and one novel gene as difference products
- Sequenced mouse and human HESR1 – identification as a bHLH transcription factor
- Generation of effective antisense molecules to HESR1
- HESR1 determined to be necessary for tube formation by blocking with antisense
- Characterization of expression pattern of known genes
- Completion of DP2 screen
- Identification of eight known genes and three novel gene as difference products
- Identification of βIg-H3, NrCAM, ESM-1, Id-2, chondroitin sulfate and annexin II as known genes potentially important in angiogenesis

- Identification of A4 and G7 as new members of the purine nucleotidase, and protein disulfide isomerase families, respectively
- Determination of tissue distribution of upregulated genes
- Demonstrated enhanced ESM-1 expression in several tumors
- Demonstrated a role for βIg-H3, and possibly NrCAM, in in vitro tube formation by blocking gene expression with antisense oligonucleotides
- Demonstrated βIg-H3 expression in tubes as well as in vessels in vivo
- Identified HESR1 as a downstream target of notch signaling
- Demonstrated a role for notch signaling in EC tube formation

## Reportable Outcomes for years 1-3

- Invited speaker at Vascular Biology '99: Symposium on Cardiovascular Genomics. "A novel bHLH zipper protein and its role in angiogenesis"
- Presentation at NAVBO 2000: First Conference on Arteriosclerosis, Thrombosis and Vascular Biology. "Downregulation of VEGFR1 and VEGFR2 by the bHLH transcription factor A21/HESR1: A potential role for A21/HESR1 in Angiogenesis".
- Presentation at Era of Hope meeting 2000 "Identification of endothelial cell genes induced during capillary angiogenesis
- MS published: "The bHLH transcription factor HESR1 regulates endothelial cell tube formation"
- MS in press: "Identification of endothelial cell genes expressed in a tube-forming model of angiogenesis: upregulation of ESM-1, βig-h3 and NrCAM "

## Conclusions

We have completed two screens at 18 hours by analyzing the DP2 pool as well as the more stringent DP3 pool generated from the RDA procedure. These screens identified several new genes upregulated during the process of EC tube formation, including the known genes HESR1, β-Ig-H3, NrCAM, annexin II, Id2, chondroitin sulfate, $\alpha_v$ and PAI. We have also identified two novel genes that appear to be new members of previously recognized gene families.

We now routinely use antisense oligonucleotides to reduce target gene expression in EC. Using this system we have shown the importance of HESR1 and β-Ig-H3 expression to in vitro tube formation. Blocking of de novo expression blocks tube formation in a dose-dependent manner, whereas nonsense sequences have no effect. Neither ESM-1 nor NrCAM appear to be essential for network formation in our current assay, however, we suspect that this assay does not model some important elements of true angiogenesis and for this reason we have developed a new assay. We have high hopes that this new system will allow us to assay the true role of the genes we have identified, particularly as we suspect some of them may have roles in budding and branching of new vessels.

The significance of our findings to date is that we have identified genes that may be useful targets in strategies aimed at blocking angiogenesis in tumors. Blocking angiogenesis is likely to inhibit, if not kill, the tumor.

## References

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Aitkenhead M, Wang S-J, Mestas J, Heard C and Hughes CCW. 2002. Identification of endothelial cell genes expressed in an in vitro model of angiogenesis: upregulation of ESM-1, •ig-h3 and NrCAM. Microvascular Research (in press).

## Appendices

Manuscript (in press)

Microvascular Research
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# Identification of Endothelial Cell Genes Expressed in an *in Vitro* Model of Angiogenesis: Induction of ESM-1, βig-h3, and NrCAM[1]

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Blood vessel growth by angiogenesis plays an essential role in embryonic development, wound healing, and tumor growth. To understand the molecular cues underlying this process we have used the PCR-based subtractive hybridization method, representational difference analysis, to identify genes upregulated in endothelial cells (EC) forming tubes in 3D collagen gels, compared to migrating and proliferating cells in 2D cultures. We identified several previously characterized angiogenic markers, including the $\alpha_v$ chain of the $\alpha_v\beta_3$ integrin and plasminogen activator inhibitor-1, suggesting overlap in gene expression between tube-forming cells *in vitro* and *in vivo*. We also found a 2- to 10-fold upregulation of βig-h3 (a collagen-binding extracellular matrix protein), NrCAM (a "neural" cell adhesion molecule), Annexin II (a tPA receptor), ESM-1 (an EC-specific molecule of unknown function), and Id2 (an inhibitory bHLH transcription factor). We identified a novel splice variant of the ESM-1 gene and also detected dramatically enhanced expression of ESM-1 and βig-h3 in several tumors. Antisense oligonucleotides to βig-h3 blocked both gene expression and tube formation *in vitro*, suggesting that βig-h3 may play a critical role in EC–matrix interactions. These data expand the suite of genes implicated in vascular remodeling and angiogenesis. 

*Key Words:* angiogenesis; gene expression; RDA; antisense.

## INTRODUCTION

Angiogenesis, the development of new blood vessels by sprouting from the preexisting vasculature, plays an important role in a number of physiological and pathological processes. Significant blood vessel growth has been shown to accompany organ development during embryogenesis, formation of the corpus luteum, placentation, wound healing, and tumor formation (Conway *et al.*, 2001; Yancopoulos *et al.*, 2000). The growth of new blood vessels accompanying tumor development has, in particular, lead to greater interest and improved understanding of the central role of the endothelial cell (EC) during angiogenesis.

After initial activation by angiogenic mediators such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), EC degrade the local basement membrane, migrate into the underly-

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ing stroma, proliferate, and form capillary sprouts. It is believed that opposing sprouts coalesce and form a new capillary loop (Folkman, 1985). However, these events are rare in normal, healthy, adult tissues and most EC are quiescent, subject only to short, tightly regulated bursts of angiogenic activity. It has become increasingly clear that these brief periods of EC activation are regulated by a balance of pro- and anti-angiogenic forces (Hanahan and Folkman, 1996; Risau, 1997). In normal adult tissues, anti-angiogenic mediators are dominant and EC are suppressed and quiescent. During angiogenesis, the release of molecules, such as VEGF, overwhelms the inhibitors and EC undergo the morphological steps necessary to generate a new capillary loop.

The molecular cues underlying these morphological changes are, in large part, unknown. Some of these events can be modeled, however, using one of a range of *in vitro* assays. These assays test the ability of putative angiogenic mediators to induce EC to migrate, proliferate, or form tube-like structures. EC embedded in type I collagen undergo rearrangement to form capillary tube-like structures (Madri *et al.*, 1988; Montesano *et al.*, 1983). These structures are morphologically similar to the primary capillary plexus that is formed after the blood island stage of embryonic development. This assay models the EC migration, alignment, and interaction with the extracellular matrix (ECM) that occurs during angiogenesis. The initial stages of angiogenesis are characterized by both matrix degradation and deposition. Collagen deposition seems particularly important: inhibition of collagen assembly and deposition prevents angiogenesis (Haralabopoulos *et al.*, 1994), while collagen type I knockout mice die *in utero* with malformed and ruptured blood vessels (Löhler *et al.*, 1984). The collagen gel model has been used extensively to study the angiogenic/anti-angiogenic potential of molecules such as insulin-like growth factor (Nakao-Hayashi *et al.*, 1992), leukemia inhibitory factor (Pepper *et al.*, 1995), VEGF-D (Marconcini *et al.*, 1999), bFGF-induced plasminogen activator (Giuliani *et al.*, 1999), and connective tissue growth factor (Shimo *et al.*, 1999). In addition, signal transduction pathways have been investigated using specific activators and inhibitors of signaling molecules (Ilan *et al.*, 1998). However, it is still the case that while the interaction of EC with ECM molecules and the activity of pro- and anti-angiogenic molecules on EC *in vitro* are well documented, considerably less is known about subsequent downstream gene expression and activation.

The aim of the present study was to identify the gene profile expressed by EC forming tubes in a 3D collagen gel. We isolated cDNA from tube-forming EC and from EC migrating and proliferating on top of a collagen gel, both grown in the presence of bFGF and VEGF, and enriched for genes differentially expressed in tubes using the PCR-based subtractive hybridization method, representational difference analysis (RDA). Our assay, therefore, selects for genes expressed in response to the combination of growth factors and extracellular environment rather than in direct response to growth factors alone. Our findings indicate that well-known angiogenic genes are differentially expressed during tube formation *in vitro*, suggesting that the 3D collagen gel assay models at least some aspects of angiogenesis. Furthermore, we have identified several genes that have not been previously implicated in angiogenesis.

## MATERIALS AND METHODS

### *Cell Culture*

Human capillary endothelial cells (HUCE or EC) were prepared from liposuction adipose tissue using anti-CD31-coated magnetic beads as described previously (Springhorn *et al.*, 1995). Tissue was obtained under protocols approved by the appropriate IRB committees. A 10-ml vial of collagen gel mixture was made by adding 1 ml 10× Hanks' balanced salts (Gibco, Grand Island, NY) to 4 ml 50 mM Hepes (pH 8.5) over ice. After the Hanks'/Hepes mix was supplemented with 30 mg fibronectin (Sigma, St Louis, MO), 5 ml of 3 mg/ml rat tail collagen type I was added to give a final solution of 1.5 mg collagen/ml. This mixture rapidly polymerizes to form the collagen gel when warmed to 37°. Rat tail collagen was prepared according to the method of Schor *et al.* (2001). In our experience, better tubes are formed in the presence

of fibronectin. To induce capillary formation, $1 \times 10^5$ HUCE were seeded onto a thin layer of collagen gel in the well of a 24-well plate, allowed to adhere for 1 h, and then overlaid with a second layer of collagen (0.5 ml) to provide a 3D matrix. Cells were grown in Medium 199 supplemented with 20% FBS, 20 ng/ml recombinant human VEGF (Genzyme, Cambridge, MA), and 20 ng/ml recombinant human bFGF (R&D Systems, Minneapolis, MN). These 3D gel EC are referred to as tube-forming EC or tubes. Cells were also grown directly on the bottom layer of collagen gel in the same medium (2D culture). Importantly, both the 2D and the 3D cells were cultured in identical medium, containing bFGF and VEGF.

### *Representational Difference Analysis*

For RDA, EC were harvested after 18 h in culture using 0.4% collagenase I (Worthington, Lakewood, NJ) and collected by centrifugation. RNA was prepared using RNA isolation kits (Stratagene, La Jolla, CA). Poly A+ mRNA was purified over oligo dT columns and reverse transcribed into double-stranded cDNA using a cDNA synthesis kit from Stratagene. Tester representation (amplified cDNA from tube-forming EC) and driver representation (amplified cDNA from 2D EC) were subjected to three rounds of subtractive hybridization exactly as described (Henderson *et al.*, 2001; Hubank and Schatz, 1999), using ratios of 1:100, 1:800, and 1:400,000.

### *Cloning and Characterization of Difference Products*

The second and third difference products (DPII and DPIII) were digested with *Dpn*II and cloned into the *Bam*HI site of pBluescript II KS+ (Stratagene). Transformants with inserts were identified by blue/white screening. To identify duplicate clones, colonies were grown on PVDF membranes using 96-well replicas (one for each round of hybridization) and the membranes were probed sequentially with inserts cut from individual clones. Doubled-stranded cDNA cut from representative clones was radiolabeled to probe virtual Northern blots containing tester and driver cDNA populations. This allowed a first estimation of differential expression between tester and driver to be ascertained. Blots were exposed to a phosphorimaging screen (Molecular Dynamics, Sunnyvale, CA) and band intensities were measured using ImageQuant software (Molecular Dynamics). After normalization to GAPDH, relative increases between tester and driver representations were calculated.

### *Northern Blotting and RT-PCR*

For Northern blotting, poly A+ mRNA was prepared from tubes and 2D cultures and resolved on standard formaldehyde gels. After crosslinking to nylon membranes, blots were hybridized to radiolabeled probes using ExpressHyb (Clontech, Palo Alto, CA) at 68° or Ultrahyb (Ambion, Austin, TX) at 42°. Multiple tissue Northern (MTN) blots (Clontech) and a matched tumor/normal tissue array (Clontech) were hybridized with ExpressHyb at 68°. After hybridization, blots were washed and developed according to the manufacturer's instructions. Blots of tubes and 2D cultures were imaged and quantified on the PhosphorImager. MTN blots were exposed to Kodak X-ray film. The tumor array was imaged and quantified on the PhosphorImager. Spot intensities for each of the probes were statistically analyzed with Kendall's rank correlation, using StatView 5.0 (SAS Institute, Inc., San Francisco, CA).

For RT-PCR, 2 $\mu$g of total RNA was prepared from tube-forming and 2D EC, random primed, and reverse transcribed with Superscript II (Gibco). Specific primers for ESM-1 (upper 5'gctaccgcacagtctcagg3' and lower 5'attgcatttttagttcttgagtgt3') and GAPDH (upper 5'accacagtccatgccatcac3' and lower 5'tccaccctgttgctgtgctgta3') were used. After a hot start at 94°, primers were annealed for 45 s (ESM-1 at 55°, GAPDH at 60°) and extended for 45 s at 72°. PCR was performed for 30 or 40 cycles.

### *Antisense Assay*

Sequence-specific antisense oligonucleotides were designed using the program "Oligo" (National Biosciences, Plymouth, MN). Two of six sequences designed for $\beta$ig-h3 are reported here: −696 5'gtggtcggctttcaggag3' and −1263 5'gtcaaccgctcacttcca3'. For the controls the following sequences were used:

VEGFR2 5′cggactcagaaccacatc3′ and nonsense control 5′ccctcccttgttactccc3′. All the nucleotides were sulfate-substituted to avoid degradation by nucleases (S-oligonucleotides). Oligonucleotides were tested at various doses ranging from 0.03 to 3 $\mu$M and their ability to block tube formation in collagen gel cultures was assayed in 24-well plates. EC were preloaded with oligonucleotides by osmotic shock using Influx (Molecular Probes). In each well, $1 \times 10^5$ EC were embedded in collagen as before. Assays were performed in triplicate and cultures were fixed in 4% paraformaldehyde/PBS 18 h after plating. After fixation, each well was scored independently by two highly skilled individuals with no prior knowledge of the arrangement of the assay and the degree of tube formation was graded in five levels from 0 to 4. The scale used was 0, monolayer; 1, some endothelial cell elongation; 2, tube formation with some branching; 3, extensive tube formation and branching; 4, extensive lattice formation. Statistical analysis was performed using a Student *t* test. To demonstrate the specificity of the antisense molecules, total RNA was extracted from the tube-forming EC and reduction in $\beta$ig-h3 mRNA levels was confirmed by RT-PCR with specific primers (upper 5′tgaccctcctggctcccctgaat3′ and lower 5′gccccgatgcctccgctaac3′) as before (see above) with an annealing temperature of 60°.

## RESULTS

### *Identification of Induced Genes*

EC were plated on top of a collagen gel or within a collagen gel sandwich and cultured in medium containing bFGF and VEGF. After 18 h, EC embedded in the 3D collagen formed an extensive network while those plated on top of collagen formed a regular monolayer (Fig. 1). In the gels EC become elongated and fused with their neighbors to form an intricate, capillary-like web. Large open spaces were visible between the cells (Fig. 1). Frozen sections cut perpendicular to the plane of the network revealed lumens in many of the cross-sections (Henderson *et al.*, 2001, and data not shown).

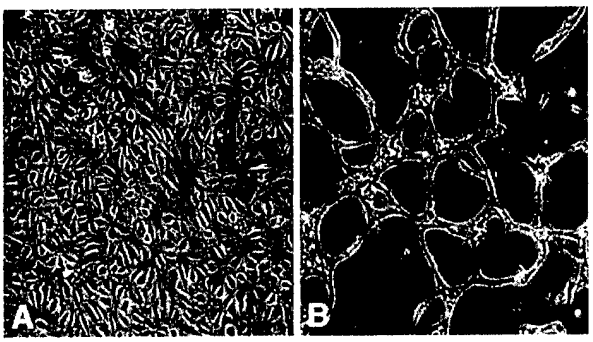


FIG. 1. EC tube formation. (A) EC grown on a layer of collagen (2D) form typical monolayers. (B) EC cultured between two layers of collagen (3D) form elongated tube-like structures. Both cultures contain bFGF and VEGF at 20 ng/ml. Sections cut perpendicular to the plane of culture revealed the presence of lumens (Henderson *et al.*, 2000, and data not shown). Original magnification ×400.

After performing three rounds of RDA, DPII and DPIII cDNA fragments were cloned into pBluescript and sequenced. It was anticipated that DPIII clones would only be expressed in the tester representation and that some genes of interest, expressed at low levels in 2D cultures and upregulated in the tube-forming EC, would be thereby excluded. To circumvent this potential problem, we decided to also examine DPII clones. Sequences were queried against GenBank using BLAST (Table 1). The most common gene isolated was spermine/spermidine N1-acetyl transferase. This housekeeping gene is involved in recycling of polyamines and is expressed by cells when they stop proliferating. Another common cDNA enriched during the RDA encoded a novel bHLH transcription factor HESR1, which has been described elsewhere (Henderson *et al.*, 2001). HESR1 is necessary for proper tube formation *in vitro* and modulates EC expression of the VEGF receptor flk-1/KDR (VEGFR2).

Most of the remaining clones identified in the DPII were isolated only once or twice in the 65 clones sequenced. Many of these clones encoded for housekeeping proteins that appear to be upregulated during tube formation. The expression pattern of this class of molecules was not examined further.

In large open-ended screens such as this, it is critical to have secondary and tertiary screens that both confirm the differential expression and reduce the number of genes to be analyzed in greater depth. We have used Northern blots and RT-PCR to confirm differen-

TABLE 1
Differentially Expressed Genes Identified by RDA

| Gene | GenBank Accession No. | No. of hits | Cloned from | Fold increase in tester over driver |
|---|---|---|---|---|
| Spermine/spermidine N1-acetyl transferase | NM_002970 | 14 | DPII/III | 7.0 |
| HESR1 | AF151522 | 12 | DPII/III | 8.0 |
| Connexin 40 | AF151979 | 4 | DPII/III | 3.0 |
| ESM-1 | NM_007036 | 4 | DPII | 4.2 |
| Very long chain acyl CoA dehydrogenase | D43682 | 2 | DPII | ND |
| KIAA0850 (NS1-BP) | XM_001739 | 2 | DPII | ND |
| Carbonic anhydrase II | XM_005208 | 1 | DPII | ND |
| $\alpha_v$ integrin | XM_005208 | 1 | DPII | 3.0 |
| Pre-chondroitin sulfate | U16036 | 1 | DPII | 3.0 |
| Endothelial plasminogen activator inhibitor | X04429 | 1 | DPII | 3.5 |
| 3-5 cAMP phosphodiesterase | XM_001862 | 1 | DPII | ND |
| Annexin II | XM_015855 | 1 | DPII | 1.7 |
| NrCAM | XM_004950 | 1 | DPIII | 9.8 |
| Cytochrome P450 (CYPa1a1) | XM_007727 | 1 | DPII | ND |
| ADAM-10 | XM_007741 | 1 | DPII | ND |
| Big-h3 | M77394 | 1 | DPIII | 5.3 |
| Id-2 | XM_002273 | 1 | DPII | 5.0 |
| General transcription factor IIi | XM_011605 | 1 | DPII | ND |
| KIAA0911 | AB020718 | 1 | DPII | ND |
| KIAA0538 | AB01110 | 1 | DPII | ND |
| Unknown (A4) | | 1 | DPII | 1.8 |
| Unknown (G7) | | 1 | DPII | 1.6 |

tial expression and then used tissue expression studies and, finally, functional assays to focus in on a small subset of genes that are likely to be of greatest interest. These studies are described below.

### *Confirmation of Differential Expression by Virtual Northern*

We next selected several genes for further analysis. As a preliminary screen to confirm differential expression of these genes, the cDNA fragments were radiolabeled and used to probe a virtual Northern of tester and driver cDNA. Blots were analyzed on a PhosphorImager and intensities of the bands normalized to GAPDH (Fig. 2A).

Several genes that have been implicated in angiogenesis were confirmed as being differentially expressed in tubes. Expression of the $\alpha_v$ integrin was 3-fold higher in the tester (tubes) than the driver cDNA. Similar increases in expression were observed with plasminogen activator inhibitor 1 (PAI-1) and Annexin II (AnnII) (3.5- and 1.7-fold increases, respectively). Levels of the glycoprotein building block, prechondroitin sulfate, were also raised 3-fold in tubes.

Some of the most significant increases in expression were observed in a group of known genes that have not previously been shown to have angiogenic function. Interestingly, NrCAM, which is important in neural development, was expressed by EC. Expression of NrCAM in tubes was almost 10 times greater than in the 2D EC. ESM-1, previously identified as being highly expressed in lung EC, was upregulated fourfold, while βig-h3, a collagen-binding protein found in vessel walls, was overexpressed fivefold in tubes. Id-2, which blocks transcription of genes containing E boxes in their promoters by sequestering bHLH transcription factors, was upregulated fivefold in tubes.

Two novel genes, A4 and G7, were also identified in this assay. Adjacent expressed sequence tags from the dbest database were aligned to form contigs of 815 bp and 2.6 kb for A4 and G7, respectively. The G7 contig is 50% homologous at the amino acid level to protein disulfide isomerase while A4 appears to be a new member of the 5′ nucleotidase family (48% homology

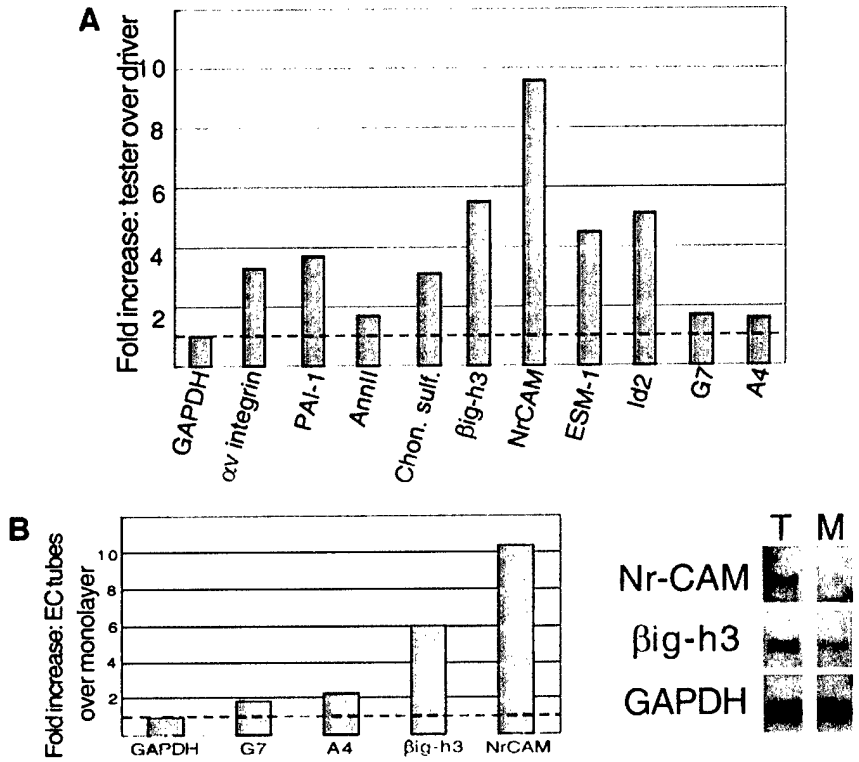


FIG. 2. Differential expression of identified clones in the tester and driver cDNA representations and on Northern blots. (A) Fold increases in expression in the tester over the driver were quantified on a PhosphorImager and normalized to GAPDH. (B) Differential expression of βig-h3, NrCAM, and the novel genes, A4 and G7, in tube-forming EC versus monolayers, was confirmed by Northern analysis. Representative Northern blots are shown for NrCAM and βig-h3. The blot was quantified on a PhosphorImager and expression was normalized to GAPDH. In both (A) and (B) the dotted line represents equal expression in monolayers and tubes.

to purine 5′ nucleotidase). Differential expression of these novel genes was 1.5 to 2 times greater in the tube cDNA compared to the 2D cultures.

### *Confirmation of Differential Expression by Northern Blot and RT-PCR*

When performing subtractive hybridization experiments and gene array studies it is important to confirm differential expression by independent assays. Therefore, we performed Northern blot analysis using mRNA from tube-forming EC and 2D cultures. We probed for βig-h3, NrCAM, and the novel genes A4 and G7 and normalized the results to GAPDH (Fig. 2B). We found excellent agreement with our virtual Northern data with the largest differences between tubes and 2D cultures detected with NrCAM and βig-h3 (10 and 5×, respectively). Again, only modest differences were observed with G7 and A4 (Fig. 2B). Northern analysis also allowed a first estimate of the transcript size of the novel genes. Discrete bands were obtained at 3.5 (G7) and 2.0 kb (A4). These genes were not analyzed further.

Differential expression of ESM-1 was confirmed by RT-PCR. At 30 cycles, two discrete bands were detectable in the sample from tube-forming EC, while nothing was visible in EC from 2D cultures (Fig. 3A). By 40 cycles, however, the same two bands were apparent in the 2D cultures (not shown). GAPDH levels were similar from both tubes and monolayers, while no-RT controls demonstrated that no genomic contamination was present. The bands corresponding to ESM-1 were sequenced and indicated the presence of a splice variant, lacking 150 bp (Fig. 3B). Sequencing of the original four ESM-1 clones obtained in the DPII revealed that one of these also had the same splice variant.

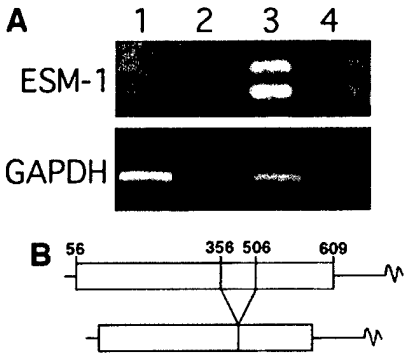


FIG. 3. Differential expression of ESM-1 and identification of a splice variant. (A) Differential expression of ESM-1 in monolayers (lanes 1 and 2) and tube-forming EC (lanes 3 and 4) was confirmed by RT-PCR. Using specific ESM-1 primers at 30 cycles, two discrete bands were observed in the sample from EC tubes (lane 3). Samples from monolayers (lane 1) and no-RT controls (lanes 2, 4) were blank at 30 cycles. Both bands from lane 3 were sequenced; the upper band contains a fragment that corresponds exactly to the published ESM-1 sequence while the lower band has 150 bp deleted. GAPDH levels were similar from monolayers and tubes (lanes 1 and 3). (B) Schematic of ESM-1 including the splice variant identified in the RT-PCR and identified in one of four ESM-1 clones in the RDA. Numbering refers to nucleotides as defined by Lassalle *et al.* (1996).

Interestingly, the open reading frame is maintained in the splice variant, suggesting that a distinct protein form of ESM-1 may be expressed, lacking an internal stretch of 50 amino acids. Future studies will determine the tissue specificity of the splice variant and whether it has a specific role to play in angiogenesis.

### *Tissue Expression of Differentially Expressed Genes*

At this point we narrowed our analysis down to βig-h3, NrCAM, and ESM-1, based on their strongly upregulated expression in tubes. To examine tissue expression of these genes we probed a MTN blot (Fig. 4). After extended exposure (9 days), bands corresponding to ESM-1 were detected in kidney and lung. All other probes were exposed for 24 h. NrCAM was strongly expressed in brain and weakly in placenta, whereas βig-h3 was detected in all tissues except brain, the highest intensities being in heart, placenta, and kidney.

Gene expression was further examined in matched pairs of normal and tumor tissue. We were unable to detect NrCAM in any tissues on the blot, suggesting that expression levels may be very low throughout the tissue, or they may be high, but only in very few cells (see discussion). There were no brain tumors represented on the blot, a tissue where NrCAM expression would be expected. ESM-1 was found to be dramatically upregulated in several (5/14) renal cell carcinomas; however, expression appeared to be somewhat higher in normal tissue in other samples (Fig. 5A). We rehybridized the blot with a probe for the housekeeping gene ubiquitin (Ub) and, using PhosphorImager data, calculated Kendall rank correlations between each probe for pooled normal and tumor specimens. As shown in Fig. 5B, no significant correlation was found between the intensity rankings for ESM-1 compared to Ub, indicating that the upregulated ESM-1 expression in tumors is not due to differences in loading of the cDNA target on the blot.

Aggressive tumors are marked by high levels of angiogenesis, which has been used as a prognostic indicator (Tas *et al.*, 2000). Similarly, high expression of VEGF by tumors has been linked to poor clinical outcome (Yuan *et al.*, 2001). In light of this we hybridized the blot with a VEGF probe and again performed correlation analysis. Here we found a very strong correlation between ESM-1 and VEGF, but again, no correlation between VEGF and Ub. Thus, ESM-1 levels

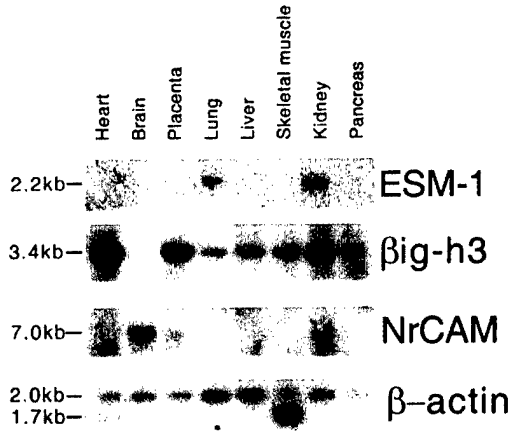


FIG. 4. Tissue distribution for NrCAM, βig-h3, and ESM-1. A multiple tissue Northern blot was sequentially probed with specific radiolabeled cDNA. All blots were exposed for 24 h except ESM-1, which was exposed for 9 days. β-Actin levels were roughly equivalent in all lanes.

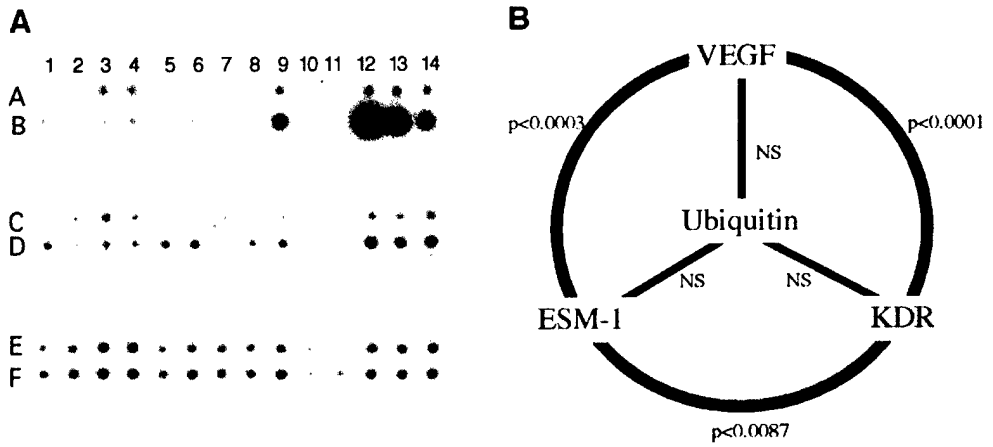


FIG. 5. (A) Expression of ESM-1 in kidney tumors. Dot blot of 14 matched normal kidney (row A) and renal cell carcinoma (row B) cDNA probed for ESM-1. Normal tissue was removed from uninvolved sites adjacent to the tumor. Note the increased expression of ESM-1 in the tumor samples in lanes 1, 9, 12, 13, and 14. The dot blot was reprobed for VEGF (rows C and D), VEGFR2 (data not shown), and ubiquitin (rows E and F). Note the similar expression pattern for ESM-1 and VEGF and the contrast with Ubiquitin. (B) Statistical correlation among ESM-1, VEGF, VEGFR2, and ubiquitin. Kendall's rank correlation determined the degree of similarity in the expression patterns of the genes in the renal cell carcinoma samples. The schematic illustrates the generated *P* values between genes. Note the strong relationship among ESM-1, VEGF, and VEGFR2, but nonsignificant (NS) correlation between these genes and ubiquitin.

correlate very well with one measure of tumor aggressiveness and vascularity. Finally, as a further measure of angiogenesis and vascularity in ESM-1- and VEGF-high tumors, we reprobed for VEGFR2 as a marker of active, angiogenic EC and with CD31 as a pan-endothelial marker. Once again we found a strong correlation between ESM-1 expression and degree of vascularity. We also found very similar correlations between ESM-1 levels and markers of angiogenesis in tumors from breast, uterus, stomach, and rectum (data not shown), indicating that ESM-1 is likely expressed in the vasculature of these tumors also. These data indicate that ESM-1 is upregulated in many tumors and that this may relate to the angiogenic state of the tumor; however, the precise role of ESM-1 in this process and the mechanistic relationship between these proteins is not known.

A similar analysis for βig-h3 showed a consistent increase in expression in the tumor samples: up in 11/14 kidney, 8/9 breast, 5/7 uterus, 7/11 colon, 8/8 stomach, and 6/6 rectum samples, with increases of up to 70-fold in some cases (data not shown). Again, there was no correlation between Ub expression and expression of βig-h3. The correlation between βig-h3 and angiogenic state (VEGFR2 expression) did not quite reach significance, likely reflecting the broader expression of βig-h3 by stromal cells (O'Brien *et al.*, 1996a), in contrast to ESM-1, which in most tissues is EC-specific (Bechard *et al.*, 2000).

### *Antisense Inhibition of Function*

To determine whether NrCAM, βig-h3, or ESM-1 are necessary for tube formation, we loaded EC with antisense oligonucleotides and assayed the cells for tube-forming ability in collagen gels. Nonsense oligonucleotides were used as negative controls and antisense oligonucleotides to VEGFR2 were used as a positive control. VEGFR2 oligonucleotides at 3 μM blocked tube formation by 75% (Fig. 6A). Neither NrCAM nor ESM-1 oligonucleotides blocked tube formation in the collagen gel assay, nor did they affect monolayer cultures (data not shown). This does not rule out a role for these genes in the more complex *in vivo* environment as not all aspects of angiogenesis are modeled in this system—for example, sprouting and anastomosis to other capillaries. Interestingly, however, antisense to βig-h3 dramatically reduced tube

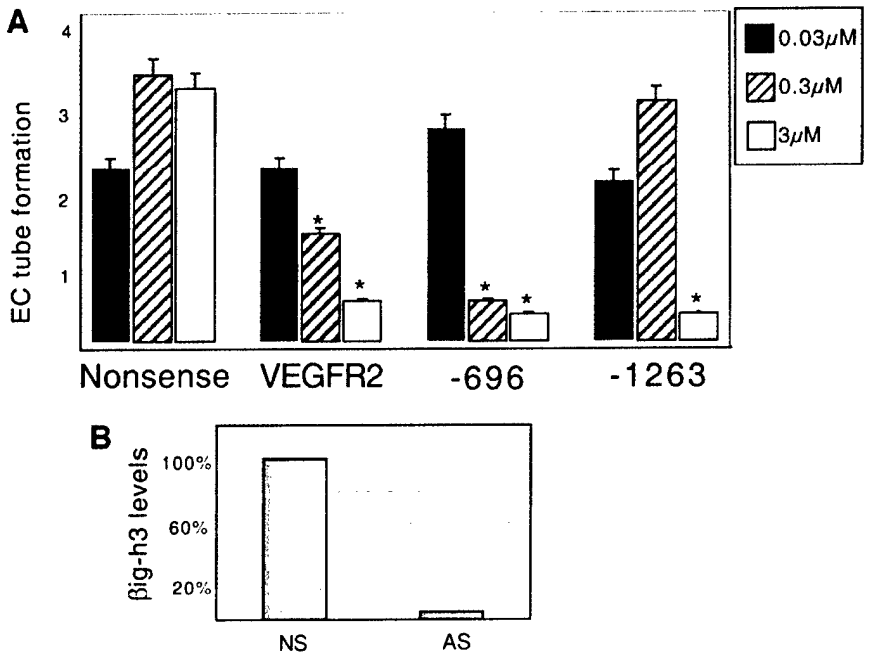


FIG. 6. Antisense blocking of βig-h3 expression. (A) Targeting βig-h3 by either antisense molecules AS-696 or AS-1263 resulted in decreased tube formation, compared to nonsense controls. VEGFR2 was used as a positive control. EC were loaded with oligonucleotides at three concentrations (0.03, 0.3, and 3 μM) and placed in collagen gels for 18 h. Tube formation was determined by two observers with no prior knowledge of the arrangement of the wells (see Materials and Methods). Each treatment was tested in three C24 wells. All results were similar, both between observers and between experiments. The results shown represent the mean scores of one observer (± standard deviation) in one of two similar experiments. *Significantly different from control, $P < 0.01$. (B) Reduction in expression of target mRNA by antisense was confirmed by RT-PCR (35 cycles) using specific primers. Band intensities were normalized to GAPDH, the expression of which was not altered by either oligonucleotide. Samples from cells incubated with 3 μM AS-696 oligonucleotide (AS) had only 5% of the βig-h3 expression compared to the nonsense control (NS).

formation in collagen gels (Fig. 6A), but had no effect on monolayer cultures (data not shown). The addition of antisense βig-h3 oligonucleotides resulted in a reduced length of EC tubes and a more poorly developed network compared to nonsense control. For both oligonucleotides shown the effect was dose-dependent, with maximal blocking of over 87% at 3 μM for oligonucleotide −696. We confirmed specific reduction of target mRNAs using RT-PCR. In EC treated with the −696 oligonucleotide, we observed a decrease in βig-h3 transcript of over 95% (Fig. 6B), whereas nonsense oligonucleotides had no effect on βig-h3 expression. Neither oligonucleotide affected GAPDH expression. Importantly, none of the oligonucleotides used in these experiments contain the GGGG motif, previously shown to be associated with nonspecific effects (Bates *et al.*, 1999).

## DISCUSSION

The collagen gel assay has been used extensively as an *in vitro* model of angiogenesis, where it has been used to study the pro- and anti-angiogenic effects of different mediators. We have used this model to identify genes that are upregulated during the early stages of tube formation as a first step to understanding the molecular basis of angiogenesis. A number of the genes we have identified have been shown previously to be angiogenic mediators, demonstrating the relevance of this model to *in vivo* angiogenesis. Moreover, these data suggest that other genes identified in this assay might also be relevant to angiogenesis. Similar screens have been performed using models that incorporate different extracellular matrix proteins and/or

different combinations of growth factors and PMA (Glienke *et al.*, 2000; Kahn *et al.*, 2000). Alternatively, tumor tissue has been sampled (St Croix *et al.*, 2000). In each case a common set of genes is isolated, which overlaps with our set, along with a suite of genes that are specific to the model being examined. This suggests that seemingly small differences between these model systems can result in subtly different suites of genes being expressed, which is in good agreement with studies showing that angiogenesis in different tissues is not necessarily equivalent (Carmeliet and Jain, 2000).

### *Previously Recognized "Angiogenic" Molecules*

AnnII and PAI-1, both upregulated in tubes, are members of a large group of molecules implicated in the plasminogen activator (PA)–plasmin pathway (Mignatti and Rifkin, 1996). AnnII provides an EC binding site for tPA and plasminogen interaction, allowing the formation of a complex that permits plasmin formation (Cesarman *et al.*, 1994; Hajjar *et al.*, 1994).

Id proteins are a family of related molecules that, by direct physical interaction, prevent bHLH transcription factors from binding to DNA (Norton *et al.*, 1998). Id2 levels were fourfold higher in tubes than in 2D cultures. A double Id1/Id3 knockout mouse is embryonically lethal with extensive vascular damage, and $Id1^{+/-}/Id3^{-/-}$ mutant mice fail to vascularize tumor xenografts, clearly suggesting a role for this class of proteins in angiogenesis (Lyden *et al.*, 1999). Interestingly, one of the most frequently isolated sequences from this screen encoded the bHLH transcription factor HESR1, and we have shown that expression of this molecule is necessary for tube formation *in vitro* (Henderson *et al.*, 2001). It is possible that Id and HESR1 may act in concert to permit EC remodeling. The integrin $\alpha_v$ was upregulated in tube-forming EC, consistent with its enhanced expression during wound healing and tumor formation (Eliceiri and Cheresh, 1999).

### *Genes Not Previously Associated with Angiogenesis*

Although initially identified as a gene upregulated in lung tumor cells by TGF-$\beta$ treatment, $\beta$ig-h3 has been independently isolated from a collagen-rich fraction of pig cartilage (Hashimoto *et al.*, 1997) and from rabbit cornea (Rawe *et al.*, 1997). This molecule appears to regulate cell to ECM interaction, as chondrocytes and fibroblasts plated onto culture dishes coated with $\beta$ig-h3 showed enhanced adhesion and spreading (Ohno *et al.*, 1999). $\beta$ig-h3 has an RGD sequence and is likely to bind the $\alpha_1$ $\beta_1$ integrin, as blocking of other integrins ($\alpha_2$, $\alpha_3$, $\alpha_5$ or $\beta_2$) has no effect on cell–$\beta$ig-h3 interaction (Ohno *et al.*, 1999). Polyclonal $\beta$ig-h3 antibodies bind ubiquitously to endothelial cells (O'Brien *et al.*, 1996b), a result that is confirmed in the present study (data not shown). Rawe *et al.* showed by *in situ* hybridization that $\beta$ig-h3 message was present in invading EC associated with corneal wound healing but not detectable on normal adult EC (Rawe *et al.*, 1997). This suggests that $\beta$ig-h3 is only actively synthesized during vessel remodeling, a proposal supported by immunohistochemical staining of tube-forming EC showing strong staining at the contact zone between the EC and the collagen gel (data not shown). By Northern blot, $\beta$ig-h3 mRNA was expressed at very low levels in monolayers but significantly upregulated in tube-forming EC. When the protein and message data are taken together, they suggest that $\beta$ig-h3 protein is present on all quiescent EC, probably bound to surface integrins, but is only actively expressed by these cells at low levels. However, once EC *in vivo* become angiogenic or migrate and begin to form tubes *in vitro*, message levels rapidly rise. It is possible that this increased expression of $\beta$ig-h3 is necessary to enhance the interaction of EC to ECM during angiogenesis. The reduction in tube formation by cells treated with antisense oligonucleotides to $\beta$ig-h3, but not with nonsense oligonucleotides, is entirely consistent with this hypothesis.

Interestingly, $\beta$ig-h3 was upregulated in over 80% of the tumors we examined (45/55), although it is likely that expression is not limited to the EC in these tissues. Together with previous publications (O'Brien *et al.*, 1996b; Ohno *et al.*, 1999; Rawe *et al.*, 1997), these data suggest a potentially important role for $\beta$ig-h3 in the interaction between angiogenic EC and the ECM.

NrCAM now joins a growing list of "neural" molecules, such as neuropilin-1, ephrin-B2, EphB2, EphB3, and EphB4, that are expressed by EC (Gale and Yan-

copoulos, 1999). Interestingly, NrCAM was undetectable in monolayers but strongly upregulated in tube-forming EC, suggesting a possible role in tube formation and angiogenesis. Wang *et al.* showed NrCAM expression in brain, pancreas, and placenta (Wang *et al.*, 1998), results largely confirmed by MTN in the present study. Recent reports that NrCAM acts as a ligand for axonin-1 suggest a role for NrCAM in neuronal outgrowth and axonal guidance (Lustig *et al.*, 1999). By extrapolation, it is tempting to speculate that NrCAM plays a role in cell-to-cell communication during the directional migration that characterizes the sprouting phase of angiogenesis—a phase not well modeled in the collagen gel assay, potentially explaining the failure of antisense molecules to block in this assay.

Although it was identified in 1996, comparatively little is known about ESM-1. Lassalle and colleagues showed that ESM-1 is an endothelial-specific, secreted protein, modulated by TNF-$\alpha$ and IFN-$\gamma$ (Lassalle *et al.*, 1996). By multiple tissue Northern analysis, ESM-1 is expressed predominately in kidney and lung; however, a more recent report indicates that ESM-1 is also expressed by the vasculature in other tissues (Bechard *et al.*, 2000). ESM-1 has been implicated in immune responses; however, our data suggest but do not confirm that ESM-1 cDNA fragments may also have a role in angiogenesis *in vivo.* Interestingly, three of four ESM-1 in the DPII pool were as published (Lassalle *et al.*, 1996) while one was alternatively spliced. Sequencing showed that the open reading frame was conserved, with identical N and C termini but possessing an internal 50-amino-acid deletion. This is the first report of an ESM-1 splice variant, although the functional significance of this is still to be determined. The deletion does not cover a recognizable motif; however, it has been recently reported that ESM-1 may be a dermatan sulfate protoglycan and that an o-glycosylation site exists at serine-137 (Bechard *et al.*, 2001), suggesting that our variant may represent a nonmodified form of ESM-1. Future experiments will be required to determine whether this variant is expressed at the protein level. A multiple tumor blot probed for ESM-1 expression revealed a dramatic increase in several tumors, including 5/14 renal cell carcinomas as well as several breast, uterine, and rectal tumors. Although not all tumors appeared to upregulate ESM-1, there was a strong correlation with the angiogenic marker VEGF, as well as with the degree of vascularity. It is possible that ESM-1 is a direct target of VEGF, although we currently have no data to support this suggestion, or it may be an independent marker of tumor angiogenesis in some tissues.

In summary, analysis of genes differentially expressed by tube-forming EC *in vitro* has revealed a suite of well-characterized angiogenic markers, as well as genes whose role in angiogenesis is previously undocumented. The appearance of well-established angiogenic markers such as $\alpha_v$ integrin and PAI-1 confirms the relevance of this *in vitro* angiogenic assay to *in vivo* systems and lends support to the assignment of other genes identified in the assay to the class of molecules relevant to angiogenesis. In particular, ESM-1, NrCAM, and $\beta$ig-h3 are interesting targets for further investigation.

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